Na^+/H^+ Antiporter, Chloroquine Uptake and Drug Resistance:
Inconsistencies in a Newly Proposed Model

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Recent publications by Sanchez *et al.*[1] and Wünsch *et al.*[2] appear to provide compelling evidence for a novel chloroquine (CQ) resistance mechanism in the malaria parasite *Plasmodium falciparum*. The authors suggest that CQ activates the Na^+/H^+ antiporter (NHE) of the plasma membrane of drug-sensitive strains, ensued by a temporary, rapid sodium–proton exchange, during which CQ itself is taken up by the antiporter into the parasite cytosol. Such activation does not occur in drug-resistant parasites, thus accounting for their diminished drug uptake and consequent resistance. Although the hypothesis has not yet been subjected to rigorous testing that only time will provide, it does seem to have been readily accepted by many students with an interest in chemotherapy.

The accumulation of CQ into malaria-infected erythrocytes is a prerequisite for its action, and CQ resistance has been almost universally associated with a reduction in the amount of drug accumulated[3]. It has been assumed that accumulation is driven by the weak base properties of CQ, ie. CQ is assumed to penetrate infected erythrocytes by passive diffusion of the free base, which becomes protonated and trapped inside the cell (presumably mostly inside the acidic food vacuole)[4]. A high-affinity and saturable component of the accumulation process was identified in the late 1960s; an intracellular receptor[5] and an active transporter or permease[6,7] have both been put forward by way of an explanation. We have recently found that it is the occupancy of this high-affinity component that correlates with antimalarial activity *in vitro*[8].

The studies of Sanchez *et al.*[1] and Wünsch *et al.*[2] clearly favor an active importer model. They provide evidence and an argument that, they claim, explain CQ resistance in terms of both physiological mechanism and altered molecular target. The principal observations to support their hypothesis are: (1) CQ uptake is temperature dependent and saturable, having a higher affinity (lower K_m) in the CQ-sensitive (CQS) strains, whereas the maximal rate of uptake was similar for both CQS and CQR strains; (2) CQ uptake was inhibited (competitively) by 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), an inhibitor of the NHE previously demonstrated in the plasma membrane of *P. falciparum*[9]; (3) CQ accumulation and its inhibition proceed in parallel with changes in cytosolic pH and sodium concentrations in drug-sensitive isolates but not CQ-resistant isolates; and (4) all of these physiological and biochemical observations are consistent with the phenotype in the genetic crosses reported by Wellems *et al.*[10]

These findings have been assimilated into a unifying hypothesis that claims that CQ accumulation and resistance in the malaria parasite are determined by the differential stimulation of the parasite NHE. In this brief comment, we would like to test the validity of these conclusions on experimental, as well as on conceptual, grounds.

Alternative interpretations for saturable uptake and temperature dependence. The fact that CQ uptake is saturable and temperature dependent does not prove that CQ is taken into the parasite by a facilitated transport mechanism. These data can equally well be explained by other widely supported theories, which assume that CQ enters the parasite merely by passive diffusion. It has long been known

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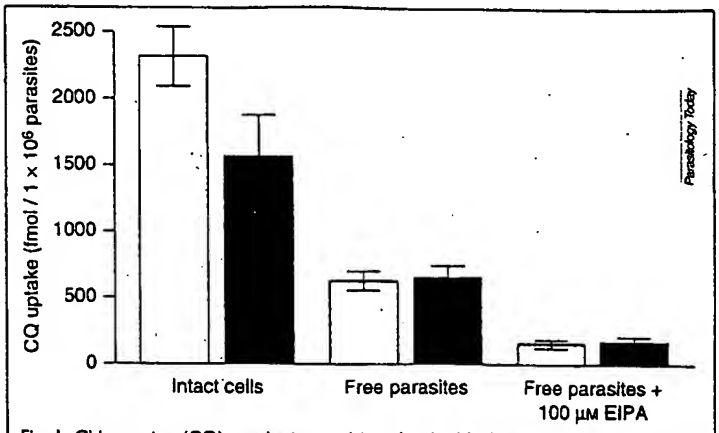

Fig. 1. Chloroquine (CQ) uptake is not driven by the Na^+/H^+ antiporter. Parasites of the HB3 CQS clone were isolated from the host cell[19]. Cells were suspended in modified Ringer solution [either 125 mM NaCl (open bars) or 125 mM CholineCl (closed bars), 10 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM D-glucose, 1.0 mM K_2HPO_4, 10 mM HEPES, pH 7.1] containing 50 nM [^{3}H]CQ (Du Pont NEN, Boston MA, USA) at 37°C for 30 min, with or without EIPA at 100 µM. Following centrifugation at 14 000 rpm for 2 min, the buffer was carefully removed using a drawn-out glass pasteur pipette. The remaining pellet was washed once in the appropriate ice-cold buffer without [^{3}H]CQ, centrifuged and the buffer removed as above. The pellet was solubilized and counted as previously described[16]. Results are means ± standard deviations of duplicate observations from five different experiments.

that CQ binds to ferriprotoporphyrin IX (FPIX), a product of parasite haemoglobin digestion. Such binding is demonstrably saturable[11]. Moreover, the generation of FPIX inside the parasite (and hence the amount of binding) relies on temperature-sensitive processes, such as the endocytic transport of haemoglobin to the food vacuole and its subsequent enzymatic cleavage. Furthermore, we have recently demonstrated that formation of the saturable component of CQ uptake, which is claimed to be a function of NHE activity, can be completely blocked by an inhibitor of plasmepsin I, the enzyme crucial to the initial cleavage of haemoglobin and therefore FPIX generation[8]. It is difficult to see how these findings could involve an NHE.

An alternative, or perhaps complementary, explanation for CQ accumulation based on the weak base trapping of CQ into acid compartments, will also be temperature-dependent and saturable as it relies on the availability of ATP to drive the vacuolar proton-pumping ATPase, and becomes saturated when the pump becomes overwhelmed[4]. Finally, it is well known that simple diffusion (of the free base in the present case) is markedly affected by temperature[12]. Hence, the employment of saturability and temperature dependence as criteria to prove facilitated (carrier-mediated) transport in a complex multi-compartmental system, such as the malaria-infected erythrocyte, is highly ambiguous.

Alternative explanations for competitive inhibition of CQ uptake by NHE inhibitors. In both reports[1,2] it is shown that inhibitors of NHE competitively inhibit the uptake of CQ, and a correlation was found between the derived inhibitory constant for some of the amiloride analogs and their relative potency as inhibitors of the antiporter as reported in the literature. However, we believe that these data may be equivocal. There is good evidence that the parasite NHE may be blocked more efficiently by using sodium-free medium than by using amiloride analogs[9]. In spite of this, the use of sodium-free medium produces a much more modest inhibition of CQ uptake into intact infected cells than the amiloride analogs, suggesting alternative or additional interactions of these compounds with the CQ uptake mechanism. We have also tested the involvement of NHE in the uptake of CQ in a more direct way: drug uptake by free parasites was found to be identical when the sodium of the extracellular medium was replaced by either choline or glucamine, ie. when the antiporter is inactive (Fig. 1)[13]. These results clearly show that the NHE is not involved in drug uptake. The authors claim to exclude the possibility that the amilorides act as weak bases that could raise vacuolar pH (ie. decrease the driving force for CQ accumulation) because their potency is not correlated with their pK_a. This seems to be a weak argument as the potency of a lysosomotropic agent does not depend only on its pK_a, but also on its rate of uptake relative to the activity of the vacuolar H^+-pump, and the ability of the protonated form to translocate across membranes, hence to dissipate the pH gradient. Methods exist for testing this presumption[14], but they have not been used. We have recently demonstrated

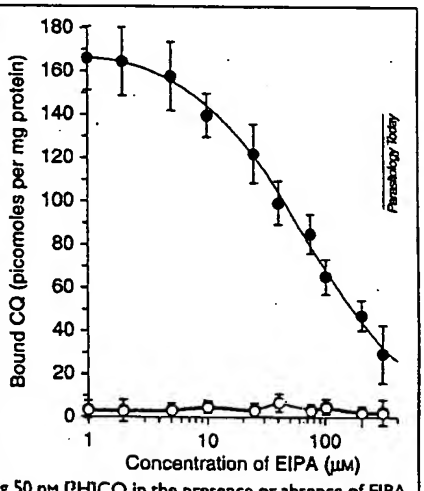

Fig. 2. EIPA displaces chloroquine (CQ) bound to ghost membranes. Erythrocyte ghost membranes were prepared by lysing washed human erythrocytes with ice-cold 5 mM sodium phosphate (pH 8.0). Membranes were centrifuged at 14000 rpm for 5 min, the supernatant was discarded and the pellet resuspended in ice-cold 5 mM sodium phosphate. This step was repeated until the membranes were white in colour. Membranes (0.27 mg protein) were loaded with FPIX or haemoglobin (5 µM) in 0.2 M HEPES in water at 37°C for 7 min. Membranes were centrifuged at 14000 rpm for 10 min, the supernatant was discarded and washed once in 0.2 M HEPES. Samples (0.01 mg protein) were incubated in 0.2 M HEPES containing 50 nM [^{3}H]CQ in the presence or absence of EIPA at the appropriate concentrations, for 10 min at 37°C. Membranes were centrifuged at 14000 rpm for 5 min. The buffer was carefully removed using a drawn-out glass pasteur pipette. The remaining pellet was washed once in the appropriate ice-cold buffer without [^{3}H]CQ, centrifuged and the buffer removed as above. The pellet was solubilized and counted as described above. Data points represent means of single observations from ten different experiments. Error bars indicate the standard deviation. Data from FPIX-loaded (closed circle) and haemoglobin-loaded (open circle) ghosts are shown.

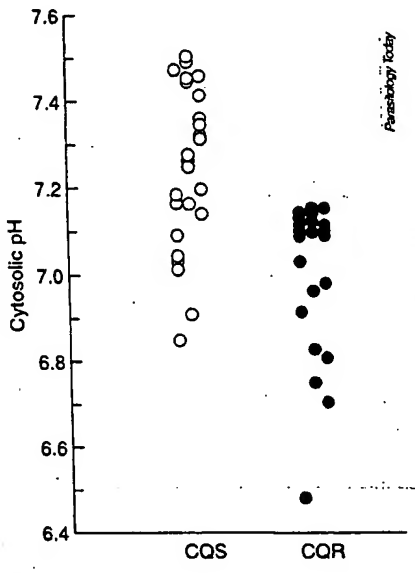

Fig. 3. Cytosolic pH of HB3 (CQS) and K1 (CQR) clones. Free parasites[22] were loaded with 1 mM BCECF-AM {2',7'-*bis*[carboxyethyl-5(6)-carboxyfluorescein]-AM, Molecular Probes, Leiden, The Netherlands} for 1 h at 37°C. The parasites were washed and resuspended in buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.2 mM $CaCl_2$ supplemented with 10 mM glucose and buffered to pH 7.4 with 10 mM HEPES) and allowed to settle on an APTS-coated glass coverslip[23]. Digital imaging microfluorimetry was carried out with a Nikon Diaphot inverted microscope (Nikon, London, UK) and a Quanticell 700 series image analysis system (Applied Imaging International Ltd, Sunderland, UK). Background subtraction was performed independently for each excitation wavelength used (440 nm and 490 nm) with emission measured above 510 nm. Fluorescence was calibrated for each cell using extracellular buffer adjusted to pH 9 and pH 5.5 in the presence of 1 mM nigericin (SIGMA). The modified Grynkiewicz equation[24] was used to calculate pH.

$$pH = pK_a + \log\frac{R - R_{min}}{R_{max} - R} + \log(F_{440\,min}/F_{440\,max}) \quad (1)$$

where F = fluorescence at the stated wavelength, pKa = 6.98, R = ratio F_{490}/F_{440}, R_{min} = ratio at pH 5.5 and R_{max} = ratio at pH 9. Data points are single observations from five different experiments. Cytosolic pH is 0.25 pH units lower in the K1 (closed circle) CQR clone (mean pH = 6.992, s.d. = 0.179, s.e.m. = 0.038, n = 22) compared with the HB3 (open circle) CQS clone (mean pH = 7.244, s.d. = 0.186, s.e.m. = 0.0372, n = 25). The difference is significant by Mann–Whitney *U*-test ($P < 0.001$).

that the saturable component of CQ uptake is due to binding of the drug to FPIX and this binding is responsible for antimalarial activity[8]. Following these findings, we have obtained strong evidence that indicates that the amilorides can bind to FPIX and displace CQ from its FPIX-binding sites in a parasite-free system (Fig. 2). Therefore, the inhibitory effect of amilorides on drug uptake into parasites could be explained by displacement of CQ bound to FPIX inside the parasite[13].

Is the movement of CQ into the parasite linked with NHE activity? Lanzer's group has put forward a rather unusual mechanistic explanation for the transport of CQ through the parasite NHE. The NHE in CQS parasites was found to be stimulated by CQ, and it was proposed that CQ is transported through the NHE in a burst of drug-stimulated sodium/proton exchange. As no similar activation could be observed in CQR parasites, the authors have suggested that the NHE of CQR parasites is constitutively and maximally activated and therefore unable to transport CQ (although the uptake of the drug by CQR parasites apparently is still inhibited by EIPA[1]). Apart from the obvious conceptual difficulties that arise from such an unusual transport mechanism, it is hard to reconcile this explanation with a variety of published data. For example, it has been demonstrated that CQ uptake can be competitively inhibited by amodiaquine (AQ) and vice versa[15]. These data suggest that the structurally similar drugs share a common mechanism of uptake. It has been reported recently that the saturable uptake of AQ into CQR parasites is considerable, being roughly equivalent to that of CQ into CQS parasites[16]. Because the NHE of CQR is already activated and capable of little, or no, further stimulation, the uptake of this closely related drug must be due to a different mechanism altogether.

The papers from Lanzer's group have concentrated on the initial rate of drug uptake (5 min), over which time drug stimulation of the NHE is complete; the NHE is at 'activated steady state' and therefore is no longer capable of transporting CQ[2]. However, we have recently demonstrated that the antimalarial activity of CQ is determined by the steady-state level of drug, which takes at least one hour to achieve[8]. The smooth and continued accumulation of CQ over one hour[2,17] indicates that the co-transport of CQ during the short NHE 'activation phase' must occur in conjunction with additional mechanisms in the CQ accumulation process.

NHE are known to have a stoichiometry of one sodium ion (Na^+) exchanged for one proton (H^+) in the case of the electroneutral variant and two Na^+ for one H^+ in the case of the electrogenic variant. Inspection of the data presented by Wünsch *et al.*[2] seems to indicate that the known tight Na^+–H^+ coupling is violated. During the first 5 min following the addition of CQ, four H^+ are transferred for each Na^+, and this ratio falls to 0.25 in the next 5–10 min period (Figs 7A and 7B in Ref. 2). Similarly, the expected kinetic correlation between the uptake of CQ and Na^+ is also violated (Figs 7B and 7C in Ref. 2). The effect of CQ on Na^+ uptake would predict an untenable increase in osmolarity of the parasite cytosol, which is inconsistent with the morphological and biochemical observations associated with CQ action (Ref. 18 and references therein).

A key observation in Ref. 2 is the reduced cytosolic pH in the CQS isolate in the absence of CQ, and the significant cytosolic alkalinization produced by IC_{50} concentrations of CQ. This was based on cytofluorometric pH measurements in random 'areas of interest' in the cytosol. We have also investigated cytosolic pH, albeit in a less extensive series of isolates. However, based on microscopic analysis, we were unable to exclude any intracellular organelle other than the food vacuole. Consequently, we have used all of the cytosol in our measurements of pH. Under these conditions, we observe much greater variability in our measured pH than that reported by Wünsch *et al.*[2] and, if anything, our CQR K1 isolate had a more acidic cytosolic pH compared to the CQS HB3 strain (Fig. 3). In addition, exposure of the HB3 parasite to CQ, at a concentration equivalent to the IC_{50} determined in our laboratories (15 nM), failed to induce any measurable alteration in pH.

Does the inclusion of the parasites from the genetic cross provide definitive proof for the NHE hypothesis? Strong support for the NHE hypothesis is the use of the progeny from the genetic cross produced by Wellems *et al.*[10] Lanzer's group demonstrated that saturable CQ uptake

was reduced and cytosolic pH increased in the resistant progeny. Both of these observations can be explained by alternative mechanisms (see above). The main phenotypic characteristics that define CQ sensitivity and resistance, in both the genetic cross and in unrelated isolates, is the ability of verapamil to enhance CQ's accumulation and activity[8,10]. Alignment of these observations with Lanzer's proposal dictates that verapamil, a known inhibitor of drug transporters and ion channels, must somehow restore the ability of the NHE of CQR parasites (which apparently is already working at maximal capacity) to be stimulated by drugs. This contrasts with recent experimental data demonstrating that the verapamil effect is independent of NHE activity in that the ability of verapamil to raise CQ accumulation in CQR parasites is retained in sodium-free medium[8]. The verapamil effect is perfectly linked with the CQ-resistant phenotype in a genetic cross[10]. Hence, if mutations of the parasite NHE are involved in CQ resistance, resistant parasites would have to evolve an independent mechanism for verapamil-stimulated drug uptake, simultaneously. This unlikely scenario suggests that mutations of the NHE are not responsible for the CQ-resistant phenotype. This reasoning is supported experimentally by the finding that, in sodium-free medium, the steady-state accumulation of CQ into CQS parasites is fourfold higher than that of CQR parasites[8].

In a further attempt to validate the NHE paradigm, Lanzer and his colleagues contended[19] that NHE is homologous to the recently identified cg2 parasite protein[20]. Mutations in cg2 were shown to be associated with CQ resistance phenotypes. However, a deeper and more critical scrutiny failed to confirm any homology between the two proteins[21].

Lanzer's group have taken care to present an intriguing alternative hypothesis to explain CQ resistance, and most importantly they have re-focused attention on CQ resistance and stimulated many people's interest. We feel that this is the correct time to highlight some of the experimental and conceptual inconsistencies that emerge from these investigations and to position this hypothesis alongside alternative explanations. It is our opinion that the NHE hypothesis has significant shortcomings. We hope that this commentary, rather than merely criticizing their data, will be the stimulus for the robust testing of all of the scientific issues raised.

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